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ETHANOL METABOLISM IN ISOLATED HEPATOCYTES

EFFECTS OF METHYLENE BLUE, CYANAMIDE AND PENICILLAMINE ON THE REDOX STATE OF THE BOUND COENZYME AND ON THE SUBSTRATE EXCHANGE AT ALCOHOL DEHYDROGENASE

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Abstract—Ethanol metabolism in hepatocytes increases the NADH/NAD^+ ratio. The mechanism was investigated by measurements of the redox state of the coenzyme bound to alcohol dehydrogenase and of ethanol–acetaldehyde exchange and concomitant hydrogen transfer between ethanol molecules. Isolated hepatocytes from fed rats were incubated with cyclohexanone and cyclohexanol or with [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]ethanol, followed by gas chromatographic determination of the redox state and isotope analysis of the ethanol by gas chromatography–mass spectrometry, respectively. Cyanamide and methylene blue decreased the redox shift caused by ethanol and increased the rates of acetaldehyde reduction during the exchange. Both drugs increased the extent of hydrogen transfer between ethanol molecules during oxidoreduction. Penicillamine had no significant effect on the ethanol-induced change in redox state of the bound coenzyme although it decreased the rate of acetaldehyde reduction. The results indicate that methylene blue inhibits aldehyde dehydrogenase and that accumulation of acetaldehyde decreases the redox effects of ethanol. The redox effect appears to result primarily from rapid elimination of acetaldehyde and equilibration with the NAD system on the alcohol dehydrogenase, but is not enhanced by further decreases in acetaldehyde concentration. Thus, penicillamine could probably be used to decrease the concentration of acetaldehyde without increasing the redox effects.

Ethanol metabolism in the liver results in redox effects which are considered to be the cause of the inhibition of gluconeogenesis [1, 2], and the production of acetaldehyde which might react with proteins to inhibit enzymatic activities [3, 4] or give rise to new antigens [5, 6]. The two primary effects may be interrelated, since rapid elimination of acetaldehyde appears to be the cause of the redox effect via the coenzyme that is bound to alcohol dehydrogenase [7]. Free NADH binds to alcohol dehydrogenase during ethanol elimination *in vivo* [8]. Thus, it was of interest to study the influence of accelerated NADH oxidation and changes in acetaldehyde elimination on the redox state of the coenzyme bound to alcohol dehydrogenase and on the rates of ethanol–acetaldehyde exchange and exchange of hydrogen atoms between ethanol molecules. These measurements were carried out with the redox indicator system cyclohexanone–cyclohexanol [7] and by studies on the formation of mono- and tetradeuterated ethanol molecules during metabolism of mixtures of [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]-ethanol [8].

Methylene blue is a drug that is considered to reoxidize NADH in the cell [1, 9, 10], and this is thought to be the reason for the counteraction of the inhibition of gluconeogenesis [1], increase in fatty acid synthesis *in vitro* [10] and inhibition of urea synthesis [11] which are caused by ethanol metabolism. Penicillamine can bind acetaldehyde *in vivo* to form a product that is excreted in the urine [12, 13], and cyanamide increases the acetaldehyde concentration by inhibiting aldehyde dehydrogenase [14].

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MATERIALS AND METHODS

Chemicals. Cyclohexanol and cyclohexanone (Merck, Darmstadt, Germany) were of GLC reference grade. [1-^{14}C]Cyclohexanone was obtained from the Radiochemical Centre (Amersham, U.K.). [1,1-$^2H_2$]Ethanol (99.6% ^2H) and [2,2,2-$^2H_3$]ethanol (99.0% ^2H) were obtained from Alfred Hempel GmbH & Co. (Düsseldorf, Germany) and [$^2H_6$]-ethanol (99% ^2H) was from Merck. Methylene blue was from GFS Chemicals (Columbus, OH, U.S.A.), D-penicillamine from Fluka AG (Buchs, Switzerland) and calcium cyanamide from American Cyanamide Company (Wayne, NJ, U.S.A.).

Hepatocyte incubations. Unfasted, female Sprague–Dawley rats (220 g) were anaesthetized with diethyl ether in the morning, and liver cells were prepared by the method of Berry and Friend [15] essentially as modified by Seglen [16]. The buffer used in the preparation and in the incubations was the bicarbonate buffer described by Krebs and Henseleit [17], containing bovine albumin (1.1 g/L, fraction V, USB Corp., Cleveland, OH, U.S.A.) and 11 mM glucose [18] equilibrated with O_2/CO_2 (19:1) and with pH adjusted to 7.4. At least 95% of the cells excluded Trypan blue [16], and the cells were used less than 30 min after preparation. The

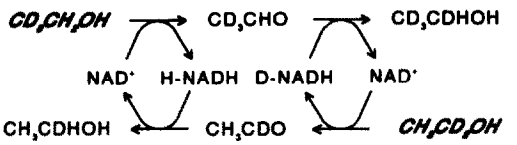

Fig. 1. Scheme explaining the formation of mono- and tetradeuterated ethanol during incubation of isolated hepatocytes with a mixture of [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]-ethanol.

cells (100 mg wet weight, corresponding to 11–14 × 10^6 cells) were incubated in a rotational shake-bath, 120 rpm, at 37° in 4 mL of buffer in stoppered vials with 7 cm^2 liquid surface area and with a 10 mL/min flow of O_2/CO_2, 19:1, through the vials [7, 19]. Drugs were added 5 min before unlabelled ethanol (20 mM) or a 1:1 mixture (20 mM) of [1,1-$^2H_2$]-ethanol and [2,2,2-$^2H_3$]ethanol. A 1:1 mixture (1 mM) of cyclohexanol and cyclohexanone was added 5 min after unlabelled ethanol. The incubations were stopped by addition of 1 mL 3 M $HClO_4$ with [$^2H_6$]ethanol as internal standard for ^2H measurements.

Analytical procedures. The samples were neutralized with 3 M KOH and centrifuged. The cyclohexanone/cyclohexanol ratio was determined by capillary gas chromatography after conversion to oxime and *t*-butyldimethylsilyl derivatives essentially as described previously [7]. The identity of the quantitated material was checked by gas chromatography–mass spectrometry [7]. The recovery of radioactivity after addition of [1-^{14}C]-cyclohexanone to quenched samples was 66 ± 11% (mean ± SD, N = 8) in the final hexane phase, and the observed ratio between the amounts of cyclohexanol and cyclohexanone after addition in a 1:1 ratio to the quenched samples was 0.98 ± 0.11 (mean ± SD, N = 10).

The concentrations of ethanols having one to four ^2H atoms were determined by gas chromatography–mass spectrometry of the 3,5-dinitrobenzoates, which were prepared and analysed with a Finnigan 4000 instrument essentially as described previously [8, 20]. The total concentration after incubation for different times with 10 min intervals were used to calculate the initial concentration and the rate of elimination by linear regression analysis. The concentrations obtained from this linear regression were multiplied by the fractions of molecules containing one to four ^2H atoms at each time point, and the concentrations obtained were used to calculate the ratio between the rates of acetaldehyde reduction and net elimination of ethanol, and the ^2H excess of the hydrogen incorporated during acetaldehyde reduction in relation to the ^2H excess in the 1-*pro-R* position of ethanol at that time [8]. This was done essentially as described previously [8], but the isotope effect was assumed to be 3.0 [8, 21]. Similar calculations were performed using the mean of the primary values from the four experiments instead of the primary values from individual experiments.

RESULTS

Metabolism of deuterated ethanols in isolated hepatocytes

Incubation of the mixtures of [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]ethanol resulted in the formation of mono- and tetradeuterated ethanol molecules. The mechanism behind this is considered to be an exchange of ^2H and ^1H between [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]ethanol during oxidoreduction via acetaldehyde as depicted in Fig. 1 [8, 20]. Thus the formation of new species indicates reversible formation of acetaldehyde.

The relative rate of the ethanol–acetaldehyde exchange and the labelling of the hydrogen used in the reduction are given in Table 1. The calculations performed using the mean of the primary values from four experiments gave values for exchange and labelling that were used to calculate the theoretical concentrations throughout the experiments [8].

Table 1. Ethanol–acetaldehyde exchange in isolated hepatocytes in the absence and presence of different drugs

Added drug	Rate of acetaldehyde reduction divided by net rate of ethanol elimination	Relative labelling of incorporated hydrogen
Methylene blue (0.6 mM)	20.8 ± 8.2*	0.96 ± 0.02*
Corresponding controls	1.1 ± 0.3	0.53 ± 0.22
Cyanamide (5 mM)	24.6 ± 18.3†	0.90 ± 0.05†
Corresponding controls	0.9 ± 0.6	0.41 ± 0.32
Penicillamine (6.7 mM)	0.20 ± 0.06†	0.48 ± 0.55
Corresponding controls	0.44 ± 0.14	0.53 ± 0.16

The parameters were obtained from the concentrations of [$^2H_1$]-, [$^2H_2$]-, [$^2H_3$]- and [$^2H_4$]ethanol at different times after addition of a 1:1 mixture of [1,1-$^2H_2$]- and [2,2,2-$^2H_3$]-ethanol (20 mM) [8].

The values are means ± SD from experiments on hepatocytes from four livers, which were the same in the corresponding control experiments.

* $P < 0.01$ and † $P < 0.05$ in comparisons with corresponding controls.

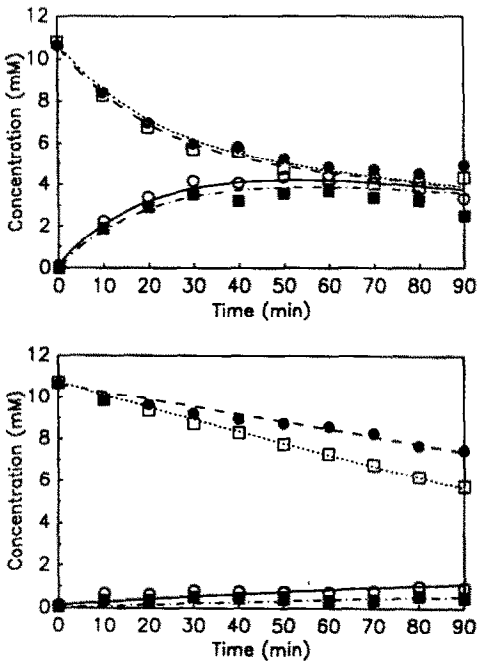

Fig. 2. Effect of methylene blue on the ethanol–acetaldehyde exchange in isolated hepatocytes. Concentrations of $[^2H_1]$ (○), $[^2H_2]$ (●), $[^2H_3]$ (□) and $[^2H_4]$ (■)-ethanol after addition of a 1:1 mixture of $[1,1\text{-}^2H_2]$- and $[2,2,2\text{-}^2H_3]$-ethanol (20 mM) to isolated hepatocytes (100 mg). Symbols show values obtained from the observed isotopic composition (mean from four experiments) and lines show the values calculated using the parameters obtained by iterative testing [8] (see Table 1). The upper panel shows the result from experiments with 0.6 mM methylene blue and the lower panel the result from the corresponding controls.

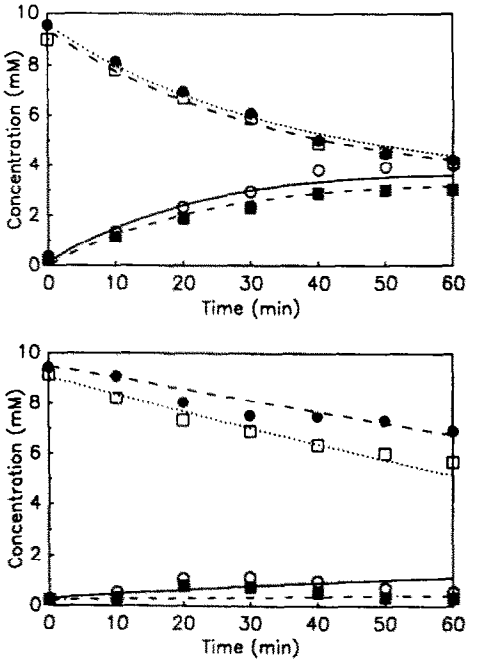

Fig. 3. Effect of cyanamide on the ethanol–acetaldehyde exchange in isolated hepatocytes. For explanations see legend to Fig. 1. The upper panel shows the result from experiments with 5 mM cyanamide and the lower panel the result from the corresponding controls.

These are compared with the values used in the calculations in Figs 2–4.

There were no significant differences between the rates of elimination in the presence or absence of the different drugs. Inhibition of aldehyde dehydrogenase with cyanamide caused a marked increase in the rate of acetaldehyde reduction. This was also seen with methylene blue, and the results with these two drugs were very similar. This was also true of the increase in relative labelling of the hydrogen incorporated during this reduction of acetaldehyde. In contrast, the addition of penicillamine decreased the rate of acetaldehyde reduction, without causing any significant change in the relative labelling of the hydrogen incorporated during this reduction.

Cyclohexanol/cyclohexanone ratio in hepatocyte incubations

The ratio between the concentrations of cyclohexanol and cyclohexanone was the same after incubation for 20 and 30 min, and closer to the initial value (1.00) after 10 min of incubation. Thus, the ratios obtained in the 20 min and 30 min incubations were used, after conversion to logarithms, to obtain

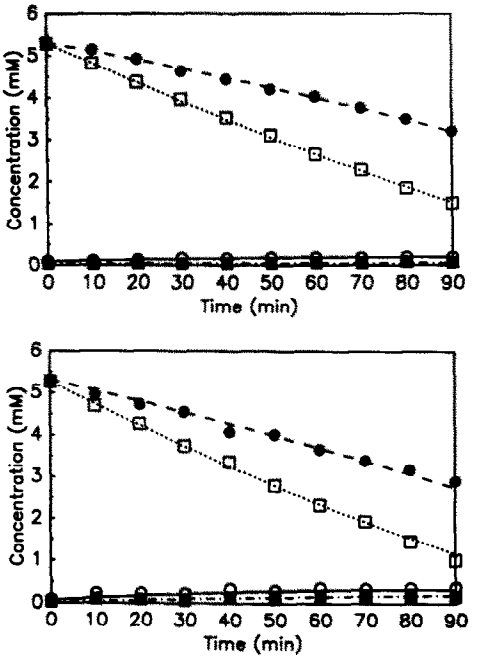

Fig. 4. Effect of penicillamine on the ethanol–acetaldehyde exchange in isolated hepatocytes. For explanations see legend to Fig. 1. The upper panel shows the result from experiments with 6.7 mM penicillamine and the lower panel the result from the corresponding controls.

Table 2. Redox shifts caused by ethanol in the presence of cyanamide and penicillamine

Added drug	Log(cyclohexanol/cyclohexanone)		Redox shift
	Without ethanol	With ethanol	
None	−1.12 ± 0.07	1.27 ± 0.30	2.39 ± 0.25
Cyanamide (5 mM)	−0.99 ± 0.11	0.46 ± 0.69	1.45 ± 0.67*
Penicillamine (6.7 mM)	−1.04 ± 0.10	0.99 ± 0.56	2.04 ± 0.66

Rat hepatocytes (100 mg) were incubated with a 1:1 mixture (0.25 mM) of cyclohexanol and cyclohexanone for 20 and 30 min in the absence and presence of 20 mM ethanol. Drugs were added 5 min before ethanol. The log(cyclohexanol/cyclohexanone) values were averaged, and the redox shift calculated as the difference between the values obtained in the presence and absence of ethanol.

The values are means ± SD (N = 4, individual rats).

* $P < 0.05$ in comparison with no addition.

Table 3. Redox shifts caused by ethanol in the presence of methylene blue

Methylene blue (mM)	Log(cyclohexanol/cyclohexanone)		Redox shift
	Without ethanol	With ethanol	
0	−1.23 ± 0.16	1.24 ± 0.10	2.46 ± 0.25
0.06	−1.38 ± 0.06	0.53 ± 0.51	1.91 ± 0.57
0.6	−1.05 ± 0.36	0.03 ± 0.09	1.07 ± 0.34

Rat hepatocytes (100 mg) were incubated with a 1:1 mixture (0.25 mM) of cyclohexanol and cyclohexanone for 20 and 30 min in the absence and presence of 20 mM ethanol. Methylene blue was added 5 min before ethanol. The log(cyclohexanol/cyclohexanone) values were averaged, and the redox shift calculated as the difference between the values obtained in the presence and absence of ethanol.

The values are means ± SD (N = 3, individual rats).

mean values (Tables 2 and 3). These were then used to calculate the redox shifts in the cyclohexanol–cyclohexanone system upon metabolism of ethanol. Addition of penicillamine did not change the redox shift, whereas cyanamide caused a decrease in the shift. Addition of methylene blue had a concentration-dependent inhibitory effect on the ethanol-induced redox shift (Table 3).

DISCUSSION

The ethanol–acetaldehyde couple has been demonstrated previously to be in a state of rapid interconversion during ethanol elimination, both *in vivo* [8] and in isolated hepatocytes [22]. This is confirmed by the present study, although the rate of acetaldehyde reduction relative to the net elimination of ethanol and the relative labelling of the hydrogen incorporated during acetaldehyde reduction were lower in the present control experiments with hepatocytes than reported previously [22] and also lower than *in vivo* [8]. This might be due to minor differences in acetaldehyde elimination by evaporation or in the feeding state of the animals [23]. The $NADH/NAD^+$ ratio on alcohol dehydrogenase, calculated from the cyclohexanol/cyclohexanone ratio, was about 400 times higher in the presence of ethanol, in agreement with previous results [7].

The aldehyde dehydrogenase inhibitor cyanamide [14] significantly decreased the redox effect of ethanol on the coenzyme bound to alcohol dehydrogenase (Table 2). The expected $NADH/NAD^+$ ratio on alcohol dehydrogenase can be calculated using the kinetic parameters given by Crabb *et al.* [24] in the equations given by Segel [25]. With an expected increase in the acetaldehyde concentration to 0.5 mM [26] the $NADH/NAD^+$ ratio on alcohol dehydrogenase can be calculated to be about 1.0 as compared to about 0.1 in the absence of substrates and cyanamide. The observed redox shift was actually about 30 times instead of 10 times, indicating that the acetaldehyde concentration was lower than 0.5 mM (Table 2). Cyanamide also caused a marked increase in the relative rate of acetaldehyde reduction in the isolated hepatocytes although the variations were large (Table 1). This increase was also seen *in vivo* [8], and at least in that case there was also a marked increase in the absolute rate of acetaldehyde reduction, indicating that the concentration of acetaldehyde in the absence of cyanamide was clearly below 37 μM, the K_m of alcohol dehydrogenase for acetaldehyde [24].

Methylene blue has been considered to inhibit the

redox effects of alcohol metabolism by increasing the rate of NADH oxidation in the cytosol by a non-enzymatic mechanism [1, 9]. This should cause an increase in the labelling of the hydrogen transferred to acetaldehyde during ethanol metabolism, since free NADH binds to alcohol dehydrogenase even during ethanol elimination *in vivo* [8]. This increase was seen in isolated hepatocytes, but it was also accompanied by a marked increase in the rate of acetaldehyde reduction (Table 1). This cannot be explained by the effect of methylene blue on the concentration of NADH, and it indicates that methylene blue acts as an aldehyde dehydrogenase inhibitor. This contention was supported by the effects of methylene blue on the redox shift caused by ethanol in isolated hepatocytes (Table 3). Thus, the effects of methylene blue both on the redox shift and on the back-reduction of acetaldehyde were similar to the effects of cyanamide. Preliminary experiments have indicated that methylene blue is actually a powerful inhibitor of aldehyde dehydrogenases from rat and human at concentrations below 0.1 mM (A. Helander, T. Cronholm and O. Tottmar, unpublished work). The inhibition of aldehyde dehydrogenase and the resulting decrease in the redox effect on the NAD system bound to alcohol dehydrogenase could conceivably explain the protective effect of methylene blue against metabolic redox effects of ethanol, such as the inhibition of gluconeogenesis [1], increase in fatty acid synthesis *in vitro* [10] and inhibition of urea synthesis [11].

Continuous administration of methylene blue in a liquid diet (0.4 μmol/mL) protects against the redox effect of ethanol in the liver [27]. Methylene blue accumulates in the hepatocytes [28] where it probably inhibits aldehyde dehydrogenase, causing acetaldehyde accumulation and attenuation of the redox effect [8, 7]. Since the persisting liver lipid accumulation in these experiments [27] may be due to the acetaldehyde, the lipid accumulation in the absence of methylene blue might still be due to a redox effect.

Penicillamine binds acetaldehyde to form a stable product [12, 13], and was therefore expected to decrease the relative rate of acetaldehyde reduction. This effect was seen in the hepatocyte incubations (Table 1). The effect was not accompanied by any change in the relative labelling of the incorporated hydrogen (Table 1) or in the redox state of the NAD system bound to alcohol dehydrogenase (Table 2). The lack of a marked increase in the redox effect is in agreement with the redox effect calculated as described above. Thus it may be concluded that penicillamine might be used to decrease the acetaldehyde concentration with no risk of enhancing redox effects.

The present results confirm that the redox effect of ethanol is due to rapid elimination of acetaldehyde [7]. They also indicate that the elimination of acetaldehyde is not normally rate-limiting, as may also be concluded from the lack of increase in ethanol elimination in penicillamine-treated animals [29].

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